An oscillating Min system in *Bacillus subtilis* influences asymmetrical septation during sporulation

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The Min system plays an important role in ensuring that cell division occurs at mid-cell in rod-shaped bacteria. In *Escherichia coli*, pole-to-pole oscillation of the Min proteins specifically inhibits polar septation. This system also prevents polar division in *Bacillus subtilis* during vegetative growth; however, the Min proteins do not oscillate in this organism. The Min system of *B. subtilis* plays a distinct role during sporulation, a process of differentiation which begins with an asymmetrical cell division. Here, we show that oscillation of the *E. coli* Min proteins can be reproduced following their introduction into *B. subtilis* cells. Further, we present evidence that the oscillatory behaviour of the Min system inhibits sporulation. We propose that an alternative Min system mechanism avoiding oscillation is evolutionarily important because oscillation of the Min system is incompatible with efficient asymmetrical septum formation and sporulation.

INTRODUCTION

Rod-shaped bacteria multiply by binary fission, in which the division septum forms with high precision at the cell’s centre. How the division machinery achieves such accuracy is a question of enduring interest. Assembly of FtsZ protomers into a circular structure, called the Z-ring, at the future division site is a prerequisite for cell division (Bi & Lutkenhaus, 1991); it is assumed that initiation of cell division is regulated at the step of FtsZ polymerization and Z-ring placement. Several FtsZ-interacting proteins modulate FtsZ polymerization, acting either to promote or to inhibit this process. MinC prevents FtsZ polymerization and acts as a direct block of polar division (de Boer et al., 1989). In min mutant strains, polar cell division results in the formation of mixtures of ‘mini’ cell forms which lack chromosomes, and extended rods containing multiple nucleoids (Adler et al., 1967; Reeve et al., 1973).

The localization and activity of MinC are dependent on interactions with MinD, an ATPase that associates peripherally with the cytoplasmic membrane (de Boer et al., 1991). MinC and MinD homologues are found in both the Gram-negative *Escherichia coli* and the Gram-positive *Bacillus subtilis*. MinD binds reversibly to negatively charged membrane lipids in an ATP-dependent manner (Hu et al., 2002; Hu & Lutkenhaus, 2003; Baráč et al., 2008). It is unevenly distributed along the length of the cell, with the highest concentration of MinD and consequently also of MinC found at the cell poles (Marston et al., 1998; Hu & Lutkenhaus, 1999; Raskin & de Boer, 1999a, b). In *E. coli*, this pattern of localization is determined by MinE. MinE tracks MinD and can be visualized as a ring-like structure at the periphery of the zone occupied by the MinCD complex at the cell pole (Fu et al., 2001; Hale et al., 2001). MinE binding to MinD is accompanied by displacement of MinC and stimulation of the ATPase activity (Hu & Lutkenhaus, 2001), leading to release of MinD from the membrane. Intracellularly, these events lead to net migration of MinD to the opposite cell pole, again followed by MinE, where the molecular events are repeated. This dynamic oscillation process, which takes place with a cycle time of 20–50 s, leads to a MinC concentration minimum at the cell’s centre, where cell division takes place (Hu and Lutkenhaus, 1999; Raskin & de Boer, 1999a, b; Fu et al., 2001; Hale et al., 2001; Juarez & Margolin, 2010; Di Ventura & Sourjik, 2011).

The Min system of *B. subtilis* features MinC (MinC<sub>Bs</sub>) and MinD (MinD<sub>Bs</sub>), but there is no MinE homologue. Instead, two proteins, MinJ and DivIVA, determine the polar localization of the MinCD complex (Edwards & Errington, 1997; Marston et al., 1998; Bramkamp et al., 2008; Patrick & Kearns, 2008). DivIVA recognizes and binds to negative

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Abbreviations: CFP, cyan fluorescent protein; YFP, yellow fluorescent protein.

Three supplementary tables and three supplementary movies are available with the online version of this paper.
membrane curvature generated at the newly forming cell poles during cell division, and it recruits the other Min system proteins so as to block the premature formation of a subsequent septum (Lenarcic et al., 2009; Ramamurthi & Losick, 2009; Eswaramoorthy et al., 2011). MinJ, a membrane protein, is recruited by DivIva to the division site that will become the new cell pole, where it accumulates and serves as a localization signal for MinD (Bramkamp et al., 2008; Patrick & Kearns, 2008).

DivIva recruits a different set of proteins to the cell poles during sporulation, when it is required for proper segregation of the axial filament, a structure that is composed of elongated sister chromosomes anchored in the vicinity of their ori regions to opposite cell poles (Wu & Errington, 1994, 1998; Webb et al., 1997). In this sporulation-specific chromosomal structure, RacA acts as a bridge between DivIva at the cell pole and the ori region of the chromosome (Ben-Yehuda et al., 2003; Wu & Errington, 2003). The implied switching of partners by DivIva may serve to couple relief of inhibition of polar septum formation to faithful chromosome segregation during sporulation. Although deletion of minD has no observable effect on the efficiency of sporulation, the sporulation septum is often misplaced closer to mid-cell in MinD-deficient cells (Barák et al., 1998; Thomaides et al., 2001). At present, the details are not known of how the inhibitory effect of the Min system proteins on polar division is overcome during sporulation.

In *B. subtilis*, oscillation of the Min proteins has not been observed, indicating a different mechanism of cell division site recognition. Although the Min system in *B. subtilis* is not as conspicuously dynamic as that in *E. coli*, there is rapid binding and dissociation of MinD(Ec) molecules at the membrane, and it is postulated that this is accompanied by MinD(Ec) polymerization and depolymerization, respectively (Barák et al., 2008). This characteristic of MinD(Ec) is not so surprising given the high sequence identity between the MinD proteins of *B. subtilis* and *E. coli* and the observation of reversible ATP-dependent membrane binding by MinD(Ec) (Drew et al., 2005). The remaining *B. subtilis* Min system proteins are less dynamic, although rapid movement of MinC(Ec) has been shown following formation of the cell division septum (Gregory et al., 2008).

The different composition and mechanism of action of the Min systems in *E. coli* and *B. subtilis* raise interesting evolutionary questions concerning (i) why different mechanisms have evolved to achieve the common goal of disabling polar division, (ii) whether the two mechanisms evolved one from another and, if so, (iii) which of the Min systems appeared first. It is known that MinD(Ec) partially complements MinD(Ec) and that YFP–MinD(Ec) expressed in *B. subtilis* localizes on helical trajectories in the same way as GFP–MinD(Ec) (Barák et al., 2008; Pavlendová et al., 2010). This indicates that MinD(Ec) is able to function together with the Min system of *B. subtilis* and more specifically, to bind to MinC(Ec) (Pavlendová et al., 2010). However, MinE is less promiscuous. It fails to form a ring-like structure or even to localize to the cell membrane of *B. subtilis*. Instead, the fluorescence signal from MinE–GFP is distributed throughout the cytoplasm, suggesting that the absence of MinE oscillation in *B. subtilis* is due to its failure to bind to MinD(Ec).

Here, we show that in the presence of MinE we can reproduce the oscillation of MinD(Ec) in *B. subtilis*. We also show that cells with oscillating MinD form spores inefficiently. This is not due to defects in signalling, as activation of the response regulator Spo0A occurs normally. Instead, it appears that the cells are affected at the stage of formation of the hallmark of sporulation—an asymmetrical septum. Sporulation would appear therefore to be incompatible with an oscillating Min system, and this may underpin the evolution of different mechanisms in the two bacterial types.

**METHODS**

**Bacterial strains, growth conditions and media.** Details of the construction of plasmids and descriptions of *B. subtilis* and *E. coli* strains used in this study are presented in Table 1 and Table S1 (available with the online version of this paper), respectively. Sequences of oligonucleotides used in this work are given in Table S2. Strains were grown in Luria broth (LB; Ausubel et al., 1987) or Difco sporulation medium (DSM; Schaeffer et al., 1965) at 37°C or as stated in the text. DNA manipulations and transformations of *E. coli* were carried out by standard methods (Sambrook et al., 1989), *B. subtilis* transformations were performed by the method of Harwood & Cutting (1990). The strains IB1230 and IB1242, with oscillating *E. coli* Min systems, tend to be unstable. These cells were always freshly prepared by transformation of chromosomal DNA from strain IB1228 into strains IB1111 and IB1112 (Table 1). When required, media were supplemented with the antibiotics ampicillin (100 μg ml⁻¹), tetracycline (10 μg ml⁻¹), kanamycin (10 μg ml⁻¹ or 30 μg ml⁻¹), spectinomycin (100 μg ml⁻¹), chloramphenicol (5 μg ml⁻¹), lincomycin (25 μg ml⁻¹) or erythromycin (1 μg ml⁻¹). Xylose at concentrations of 0.05–0.5% (w/v) was used for induction of Pxy; for induction of expression from phosphosynthase, 0.1–1 mM IPTG was used.

**Western blotting.** The intracellular levels of GFP, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fusion proteins were determined by Western blot analysis with an anti-GFP antibody (Roche Diagnostics) as described previously (Barák et al., 2008). The expression of Spo0A was detected with polyclonal anti-Spo0A antibody. After reaching the stationary phase of growth, cells were collected and processed as described previously (Barák et al., 2008).

**Fluorescence microscopy.** Cells were grown to the desired phase and a small amount of culture was transferred to microscope slides covered with a thin layer of 1% agarose in LB medium. When necessary, cells were concentrated by centrifugation (3 min × 2.3 g) and resuspended in a small volume of supernatant prior to the examination. To visualize the cells and septal membranes, the cell cultures were stained with FM 4-64 dye (Molecular Probes) at a concentration of 1 μg ml⁻¹. Fluorescence microscopy images were acquired using an Olympus BX61 microscope, equipped with an Olympus DP30BW camera and a spinning disc VivaTome Zeiss microscope. Olympus CellP imaging software and AxioVision 4.8.2.0 software were employed for image acquisition and analysis,
and the Huygens Essential software package was used for image deconvolution.

**Sporulation efficiency.** The sporulation efficiency was determined essentially as described in Harwood & Cutting (1990). Briefly, cultures were grown in DSM sporulation medium supplemented with 0.5 mM IPTG, 0.5% xylene and half the dose of appropriate antibiotics at 37 °C for 24 h after inoculation. After heat treatment (85 °C, 15 min), cells were diluted in LB medium and plated onto LB agar plates. Colonies formed from outgrowing spores on these plates represent cells that were able to sporulate and thus survive the heat treatment. These experiments were repeated at least three times. The sporulation efficiency was defined in terms of c.f.u. as follows: (c.f.u. treatment. These experiments were repeated at least three times. The sporulation efficiency was defined in terms of c.f.u. as follows: (c.f.u. strain, which was taken as 100 %.

**Bacterial two-hybrid system.** Fusions of *E. coli* MinC, MinD and MinE proteins to the T25 and T18 fragments of adenylate cyclase were constructed in the bacterial adenylate cyclase-based two-hybrid (BACTH) system (Karimova et al., 2002). To amplify genes of interest, the primer pairs minCecB2HS and minCecB2HE, minDecB2HS and minDecB2HE or minEecB2HS and minEecB2HE were used with chromosomal DNA from *E. coli* MM294 strain as template (Meselson & Yuan, 1968). Amplified genes were cloned into the EcoRI and BamHI sites of plasmids pKT25 or pKNT25 and pUT18C or pUT18. Plasmids with T25 and T18 fusions to *B. subtilis* minC, minD, minI and divIVA were a kind gift from Dr Richard Daniel, Newcastle University, UK. To test for protein–protein interactions, transformants of *E. coli* BTH101 (adenylate cyclase-deficient strain) were plated onto LB plates supplemented with 40 μg X-Gal ml⁻¹, 0.1 mM IPTG, 100 μg ampicillin ml⁻¹ and 30 μg kanamycin ml⁻¹, and grown for 24–72 h at 30 °C. To detect interactions, the BACTH system protocol was followed.

**Quantitative β-galactosidase assay.** β-Galactosidase activity was measured as described by Miller (1972) with an extra wash step added. To eliminate error due to the effects of different carbon sources in the growth medium, the cells were pelleted and resuspended in an assay buffer prior to further processing.

## RESULTS

### *E. coli* MinD oscillation in *B. subtilis*

Through a series of genetic manipulations and adjustments to growth conditions, detailed below, we have been able to generate Min system oscillation in *B. subtilis*. This phenomenon is observed in the majority, if not all, of the cells in the population and occurs with an oscillation cycle time similar to that observed in *E. coli* (Fig. 1a, Movie S1).

MinDEc does not oscillate in the absence of MinE in *E. coli* (Hu & Lutkenhaus, 2001), nor does it do so when introduced into *B. subtilis* (Pavlendová et al., 2010). We therefore examined the effect of introducing MinDEc together with MinE into *B. subtilis* by constructing strains expressing yfp–minDEc and minE in a wild-type (IB1229) and a minDBs deletion (IB1230) background. In many cells, we observed YFP–MinDEc foci close to the cell membrane, especially in strain IB1229. Movement of these ‘dots’ was generally confined to a small local region (Movie S2), and occasionally the dots relocated towards one of the cell poles. In IB1230 cells, YFP–MinDBs movement reminiscent of oscillation in *E. coli* was visible, especially in shorter cells.
Since overexpression of Min proteins causes cell elongation (Marston & Errington, 1999; Pavlendová et al., 2010), the longer cells exhibiting the brightly fluorescing dots are likely to have higher YFP–MinDEc concentrations. Higher concentrations of MinD Ec and MinE may interfere with the function of the Min system by biasing the proportions of the complexes formed. In addition, interaction among *E. coli* and *B. subtilis* Min system components may cause slower movement of YFP–MinD Ec. In *E. coli*, the period of the Min oscillation cycle is 20–50 s (Raskin & de Boer, 1999a; Touhami et al., 2006). To compare the oscillation times in *E. coli* and *B. subtilis*, we timed the YFP–MinD Ec oscillation cycle in *E. coli* strain ΔminCDE P_{lac}::yfp–minD Ec::minE–cfp (YLS1::pYLs68) (Shih et al., 2002). In our hands, oscillation was observed with a period of about 1 min at room temperature. In contrast, the oscillation of YFP–MinD Ec in *B. subtilis* ΔminD Bs yfp–minD Ec minE (IB1230) cells was slower at 1.5–3.5 min per cycle. Increasing the temperature to 30 °C, a change that in *E. coli* results in faster oscillation (from a cycle time of 20 s at 22 °C to 8 s at 30 °C; Touhami et al., 2006), did not significantly enhance the oscillation frequency of YFP–MinD Ec in *B. subtilis*. We reasoned that the presence of *B. subtilis* DivIVA or MinJ might be limiting the mobility of YFP–MinD Ec. To test this idea, we produced YFP–MinD Ec and MinE in a *B. subtilis* strain in which either minD and divIVA (ΔminD Bs ΔdivIVA yfp–minD Ec minE, IB1242) or minD and minJ (ΔminD Bs ΔminJ yfp–minD Ec minE, IB1363) were deleted. In these cells, the period of the oscillation cycle was essentially unchanged (1.5–3 min), but oscillation was observed in almost all cells (Fig. 1a, Movie S1).

Next we explored the possibility that the lower frequency of YFP–MinD Ec oscillation in the *B. subtilis* system was caused by perturbations in the concentration ratios of the Min proteins. In the *B. subtilis* strains described here, YFP–MinD Ec and MinE were expressed from the P_{hyperspansk} and P_{xyl} promoters, respectively, while in *E. coli* YLS1::pYLs68, both genes were transcribed from the P_{lac} promoter. To compare MinDE expression levels in *B. subtilis* and in *E. coli*, we performed Western blot analysis. It is...
possible to visualize both MinDEc and MinE on one blot using a monoclonal anti-GFP antibody, in a strain where both MinDEc and MinE are in fusion with fluorescent proteins (ΔminDBs yfp–minDEc minE–gfp, IB1155). Under induction conditions similar to those used for the microscopy experiments (0.5 mM IPTG and 0.1 % xylose), it can be seen in Fig. 1(c) that while the concentrations of YFP–MinDEc (upper bands in lanes 1, 2, 3, 5 and 6) are similar in both systems, the concentration of MinE–CFP (lower band, lane 6) in E. coli strain ΔminCDE Plac::yfp–minDEc::minE–cfp (YLS1::pYLS68) is higher than the concentration of MinE–GFP in B. subtilis cells, which became darker coloured as the cells sporulated. This suggested that IB1230 cells were impaired in sporulation. We therefore measured the sporulation efficiency of strain IB1230 (Table 2). The sporulation efficiency of the strain expressing both YFP–MinDEc and MinE–GFP (IB1155) (lower band, lanes 1, 2 and 3). Although significant differences in the MinE–GFP expression levels under the three induction conditions tested (Fig. 1c, lanes 1, 2 and 3) were not observed, induction with 0.1 % xylose led to the highest YFP–MinDEc oscillation frequency, which approached one oscillation period per minute in many cells of ΔminDBs (IB1230) and ΔminDBs AdivIVA (IB1242) B. subtilis strains. These experiments show that in the presence of MinE, YFP–MinDEc oscillates in B. subtilis and that the characteristics of the oscillation process closely reproduce the oscillation behaviour of the Min system observed in E. coli.

Dynamic MinD inhibits sporulation

Over several days on DSM agar plates, colonies formed by strain ΔminDBs yfp–minDEc minE (IB1230) remained brighter coloured than those formed by wild-type B. subtilis cells, which became darker coloured as the cells sporulated. This suggested that IB1230 cells were impaired in sporulation. We therefore measured the sporulation efficiency of B. subtilis cells expressing the E. coli Min proteins. Interestingly, the sporulation efficiency of strain IB1230 is 10-fold lower (9%) than that of wild-type cells sporulated. This cell, which became darker coloured as the cells sporulated. The simplest explanation for the decreased sporulation efficiency of strain IB1230 (ΔminDBs yfp–minDEc minE) is that interactions with MinDEc (Fig. 2) induce oscillation of MinCBo leading to increased MinC concentrations at the cell poles which prevent asymmetrical septation. However, the sporulation efficiency of strain IB1370 (ΔminCBo ΔminDBs yfp–minDEc minE), in which both MinDBs and MinCBo are deleted, is even lower (2%) than that observed in strain IB1230 (ΔminDBs yfp–minDEc minE). Thus, MinC

Table 2. Sporulation efficiency of B. subtilis strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sporulation efficiency</th>
<th>Oscillation</th>
<th>minDBs</th>
<th>minCBo</th>
<th>minDEc</th>
<th>minE</th>
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<tr>
<td>PY79</td>
<td>100 %</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>–</td>
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<tr>
<td>IB1056</td>
<td>85 ± 1.9 %</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>–</td>
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<tr>
<td>IB1371</td>
<td>88.8 ± 0.9 %</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>IB1111</td>
<td>85.4 ± 1.9 %</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>IB1107</td>
<td>56.0 ± 12.0 %</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>IB1229</td>
<td>53.4 ± 17.5 %</td>
<td>+/–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>IB1230</td>
<td>8.8 ± 2.5 %</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IB1370</td>
<td>1.7 ± 0.9 %</td>
<td>+</td>
<td>–</td>
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Min system oscillation blocks sporulation

Oscillating Min proteins block sporulation by inhibition of polar septum formation

The master regulator of sporulation initiation is Spo0A, a response regulator that is phosphorylated by a multi-component phosphorelay (Hoch, 1993; Perego & Hoch, 2002). Phosphorylated Spo0A binds to specific promoter regions (‘0A boxes’), and activates or represses the expression of scores of genes required for sporulation (reviewed by Piggot & Losick, 2002; Barák et al., 2005). To test whether strains exhibiting oscillation of MinD_{Ec} are defective in sporulation initiation, we examined Spo0A expression levels by Western blotting (Fig. 3a). Spo0A is present at similar levels in strain ΔminD_{Bs} yfp–minD_{Ec} minE (IB1230), which exhibits Min system oscillation, and in the wild-type strain (PY79), which does not. Indeed in all strains examined, Spo0A was detected at normal levels, the exception being the control strain in which spo0A has been deleted (IB220; Schmeisser et al., 2000). Thus the reduced sporulation efficiency associated with the oscillating Min system is not caused by perturbations in the level of Spo0A. Since spo0A expression is positively autoregulated (Molle et al., 2003), normal Spo0A levels indicate that the activity of Spo0A, and the system of proteins that activate it, is unaffected by the oscillating Min system.

A more likely explanation for the lowered sporulation efficiency is a defect in polar cell division. Oscillating MinD_{Ec} is expected to bind to MinC_{Bs}, thus conferring pole-to-pole oscillation on the cell division inhibitor, which would prevent polar septum formation. This hypothesis was tested by membrane staining. Cells of the wild-type strain (PY79) and strain ΔminD_{Bs} yfp–minD_{Ec} minE (IB1230) were grown until hours 2 and 4 of sporulation, and the membranes were stained with the dye FM4–64. The pattern of staining defined three discernible cell classes: (i) cells with a polar septum (stage II), (ii) cells in the later stages of sporulation (stage III and later), and (iii) vegetative cells.

For the wild-type strain, after 2 h, 44 % of the cells had not entered into sporulation, 49 % of cells showed a clear polar septum and the remaining cells were in stage III or later (Fig. 3b). Cells of strains ΔminD_{Bs} yfp–minD_{Ec} minE (IB1230) and ΔminC_{Bs} ΔminD_{Bs} yfp–minD_{Ec} minE (IB1370), which harbour the oscillating E. coli Min system components, were noticeably impaired in the formation of asymmetrical septa. In the second hour of sporulation, forespores in stage III or later were not observed, and an asymmetrical septum was observed in only about 27 % of the cells (IB1230 and IB1370). As mentioned previously, the sporulation efficiency of strain ΔminD_{Bs} yfp–minD_{Ec} minE (IB1230) is around 9 %. This indicates that even though polar septa are forming in 27 % of these cells at hour 2 of sporulation, only one-third of these give rise to resistant spores. In summary, B. subtilis cells, in which the E. coli Min system components oscillate, initiate sporulation normally but are impaired in sporulation septum formation.
The cells were classified into three groups. First, cells with sporulation, and the membranes were stained using FM4-64 dye. Second, cells with asymmetrical septa, representing stage II of sporulation (black); and third, cells with a clear minicell phenotype were excluded. The dynamics of MinD localization and reversible membrane binding are integral to the function of both Min systems. The determinant of MinD affinity for the membrane is an amphipathic α-helix at its C terminus (Hu & Lutkenhaus, 2003; Szeto et al., 2003). MinD<sub>Ec</sub> preferentially binds to membranes enriched in negatively charged lipids, such as phosphatidylglycerol, which are helically arranged (Barák et al., 2008). MinD<sub>Ec</sub> also oscillates on a helical trajectory, although it is not known whether helical phosphatidylglycerol domains exist in the cytoplasmic membrane of E. coli (Shih et al., 2003). The phospholipid composition of the membranes of E. coli and B. subtilis is strikingly different. Phosphatidylglycerol represents 40 and 20 % and cardiolipin 24 and 4 % of the membrane phospholipids in B. subtilis and E. coli, respectively (Kusters et al., 1991; López et al., 1998).

These comparisons raise many interesting questions, including whether the E. coli Min system would oscillate following its transplantation into B. subtilis. Elsewhere, oscillation of MinD from Gram-negative Neisseria gonorrhoeae was observed in E. coli (Ramírez-Arcos et al., 2002). Oscillation is an intrinsic property of the Min proteins of E. coli, as shown by the elegant studies on flat membrane systems (Loose et al., 2008). Here we have shown that the E. coli Min system behaves dynamically in Gram-positive B. subtilis. We discovered conditions under which E. coli MinDE oscillation in B. subtilis closely resembles oscillation in E. coli. Oscillation of the Min system proteins is therefore not restricted by the different membrane compositions of E. coli and B. subtilis. This prompts the subsidiary question of why separate mechanisms have evolved to achieve the same goal. One reason could be the incompatibility of Min system oscillation with sporulation. We observed a significant decrease in the sporulation efficiency of B. subtilis cells in which oscillation of E. coli MinD was observed. The defect is not manifested at the stage of sporulation initiation, since expression and activation of the master regulator of sporulation, Spo0A, are unaffected. In contrast, the capacity of the cells to form intact polar septa was impaired, and this was also observed in a strain in which both MinD<sub>Es</sub> and MinC<sub>Es</sub> were depleted. Taken together, these results demonstrate that expression of heterologous, oscillating Min proteins restricts polar septum formation by a mechanism that is MinC-independent.

For sporulation to occur there has to be a mechanism for liberating the polar septation sites from the division-inhibitory activity of the Min system. A key factor at this

**DISCUSSION**

Regulation of cell division site placement is an intensively studied phenomenon in the model organisms E. coli and B. subtilis. The Min system serves in both classes of organisms as an efficient blockade of unwanted polar septation, but quite different mechanisms of Min system action are postulated. In E. coli, pole-to-pole oscillation of MinCDE creates a concentration gradient of the cell division inhibitor MinC, with the highest concentration at the cell poles, where septation is restricted (Marston et al., 1998; Hu & Lutkenhaus, 1999; Raskin & de Boer, 1999a, b; Hale et al., 2001). In contrast, the MinCDJ−DivIVA complex localizes at the newly formed cell poles and persists at the polar positions in B. subtilis (Edwards & Errington, 1997; Marston et al., 1998; Bramkamp et al., 2008; Patrick & Kearns, 2008, Eswaramoorthy et al., 2011).
stage is DivIVA, with its alternative functions in vegetative cell division and in sporulation. We speculate that upon binding to RacA, DivIVA loses its capacity to bind to the Min proteins and confine them to the cell poles. This de-localization of the Min proteins would then allow SpoIIIE-dependent assembly of FtsZ-rings (Z-rings) at the site of asymmetrical septation. The presence of the oscillating Min system, transplanted from *E. coli*, has a negative effect on either asymmetrical septum formation or the later stages of the sporulation process, or on both.

**Evolution of Min systems and sporulation**

The evolutionary implications of these observations are that bacteria which form endospores will have DivIVA/MinJ rather than MinE as the auxiliary component(s) of MinCD. Until recently, sporulation was thought to be restricted to species of Gram-positive bacteria. As shown in Table S3, the genomes of all Gram-positive endospore-forming bacteria encode a DivIVA homologue and most also encode a MinJ homologue. Interestingly, most of the sporulating *Clostridiae* sp. also encode a MinE homologue (Table S3). However, it is not known whether these MinE proteins are functional, if they are part of Min systems which oscillate, and what, if any, interplay there is with the DivIVA/MinJ system during vegetative growth and sporulation.

The chromosomes of almost all rod-shaped Gram-negative bacteria encode a MinE homologue, and some encode homologues of DivIVA (*Rothfield et al.*, 2005; Table S3). Gram-negative bacteria have hitherto been considered to be non-sporulating, with the possible exception of a sparsely documented example in *Thermus*. In addition, *Mycobacterium* forms spores by converting the rod-shaped vegetative cell into a spherical spore without prior asymmetrical division (Kaiser, 2003).

From the available data it is hard to deduce which Min system evolved from which, just as we do not know whether the common ancestor of Gram-positive and Gram-negative bacteria possessed these different characteristics. We can speculate that the Min systems either evolved separately or, more likely, evolved together in Gram-positive bacteria for the alternate life cycles of vegetative growth and sporulation, as MinE and DivIVA/MinJ are present in most *Clostridiae* sp. If this assumption is true, then most probably Gram-negative bacteria evolved from a Gram-positive bacterium. This notion is supported by the recent fascinating description of the cell membrane structures of *Actinobaculum longum* (evolutionarily a close relative of *Clostridiae* sp.) during sporulation and spore outgrowth (*Tocheva et al.*, 2011). Those authors show that during sporulation the inner membrane of the mother cell is inverted and transformed to become an outer membrane of the germinating cell. Their results point to sporulation as a mechanism by which the bacterial outer membrane could have arisen. If *A. longum* is the missing link between single- and double-membraned bacteria, it is not surprising that it possesses the two cell-division regulatory systems that characterize Gram-positive and Gram-negative microorganisms. Further work is needed to address whether and how these two systems function together in the same cell.

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